AFRL-SR-BL-TR-98-

0165

REPORT DOCUMENTATION PAGE

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	28 Jan 1998	Final 6/1994 - 8/1997

4. TITLE AND SUBTITLE

Induction of Chemical Biomarkers in Fish

5. FUNDING NUMBERS

Grant - AFOSR # F49620-94-1-0264

2312/AS

61102F

6. AUTHOR(S)

John J. Lech, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Medical College of Wisconsin
8701 Watertown Plank Road
Milwaukee, WI 53226

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

AFOSR - Bioenvironmental Sciences
111 Duncan Ave.
Bolling AFB, D.C. 20332-001

NL

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

NA

12a. DISTRIBUTION/AVAILABILITY STATEMENT

Approved for public release; distribution unlimited.

19980219 126

13. ABSTRACT (Maximum 200 words)

The primary focus of this research was to develop biomarkers for the detection of estrogenic chemicals in aquatic environments. Vitellogenin (Vg) and its corresponding mRNA have been shown to be induced by estrogenic chemicals in aquatic species. In this investigation the probe, pSG5Vg1.1, was produced for detection of Vg expression in trout. Using northern blotting techniques, pSG5Vg1.1 was able to detect increases in liver Vg mRNA in fish treated with estradiol, a known inducer of Vg in fish. Nonylphenol (NP) and DDE, which are also thought to be estrogenic, were also shown to induce trout liver Vg mRNA as detected by pSG5Vg1.1. In order to investigate the potency of NP with respect to lethality and estrogenicity, in vivo concentration-response relationships for both death and Vg mRNA induction were investigated in trout. Trout were exposed to various concentrations of NP after which the estrogenic effects were determined by RT-PCR responses of liver Vg mRNA. The LC_{50}, of NP was 193 ppb. Estrogenic effects, as evidenced by the appearance of Vg mRNA, could be seen at as low as 10 ppb of NP. This research illustrates the utility of RT-PCR and DNA probes in detecting low levels of Vg mRNA induced by NP and possibly other estrogenic chemicals. These observations indicate that the potency of NP for its estrogenic effect is much greater than its lethal effect and that NP is apt to be estrogenic in the environment at concentrations which are not lethal.

14. SUBJECT TERMS	15. NUMBER OF PAGES
estrogenic chemicals; nonylphenol, fish, molecular biomarkers	5
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
unclassified	unclassified	unclassified	SAR

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std 239-18
298-102

DTIC QUALITY INSPECTED 2

Final Technical Report
AFOSR Grant #F49620-94-1-0264
Induction of Chemical Biomarkers in Fish
May 1994 - August 1997

John J. Lech, Ph.D.
Professor
Department of Pharmacology and Toxicology
Medical College of Wisconsin
8701 Watertown Plank Road
Milwaukee, WI 53226

A. Detection of Estrogenic Responses of Vitellogenin on mRNA

1) Development of a cDNA probe

Estrogenic compounds have been shown to induce specific endocrine systems in various species. The protein vitellogenin (Vg) and its corresponding mRNA have been shown to be induced by estrogenic chemicals in aquatic species, but specific probes for the measurement of this induction are not available. In this investigation the probe, pSG5Vg1.1, was produced for detection of Vg expression in rainbow trout. The probe was made by the construction of a Vg cDNA fragment which was generated by the reverse transcriptase polymerase chain reaction (RT-PCR) and placed into the plasmid, pSG5. Using northern blotting techniques, pSG5Vg1.1 was able to detect results in liver Vg mRNA in fish treated with estradiol, a known inducer of Vg expression in aquatic species. Nonylphenol and DDE, which are also thought to be estrogenic, were also shown to cause induction of trout liver Vg mRNA as detected by pSG5Vg1.1. These results indicate that Vg mRNA in fish liver induced by estrogenic chemicals can be detected using the cDNA probe, and both DDE and nonylphenol have estrogenic effects which can be detected by pSG5Vg1.1.

2) Lethality and estrogenic potency of nonylphenol

Although alkylphenols, including nonylphenol, are estrogenic and considered hazardous to aquatic life most of the work done on these compounds have been done in cell culture or other *in vitro* systems. In order to make realistic evaluations of this hazard on a quantitative basis, *in vivo* assessments of toxicological efforts should be made. Since nonylphenol is lethal at certain concentrations in water it would be of value to know the relationship between the lethal concentration and the concentration at which estrogenic effects are present.

Obviously if the concentrations needed to produce estrogenic effects are similar to lethal concentrations, then lethality would obscure the estrogenic effects. On the other hand the further away the estrogenicity curve is from the lethality curve the more important the estrogenic effects of nonylphenol would be in the environment.

In order to investigate the potency of nonylphenol with respect to lethality and estrogenicity *in vivo* concentration-response relationships for both death and vitellogenin induction, were investigated in rainbow trout. The trout were exposed to various concentrations of nonylphenol in a flow through system after which the estrogenic effects were determined by RT-PCR responses of liver vitellogenin mRNA.

The lethality of nonylphenol was tested under flow through conditions for 72 hours with a concentration range of 0 to 250 ppb (w/v). Observations were made every 24 hours with the results applied to the Trimmed Spearman-Karber statistical method. The LC_{50}, within a 95% confidence limit, was 193.65 ppb of nonylphenol.

Rainbow trout were exposed to 50 ppb of nonylphenol during an eight day time course study under flow through conditions. Sacrifices were made at 0,1,2,4 and 8 days to check for the appearance of nonylphenol induced vitellogenin. Following total RNA purification, vitellogenin mRNA bands were generated by RT-PCR from nonylphenol treated rainbow trout. The band of interest was vitellogenin (Vg)mRNA with 827 bp (Vg59). No Vg band appeared in the control

samples. The appearance of vitellogenin in RNA could be identified at 2 days with the strongest qualitative signal at 4 days. Rainbow trout were exposed to either 50 ppb nonylphenol in DMFA (treated) or to DMFA alone (Control) for 72 hours under flow through conditions. At 72 hours the fish were placed in clean water and sacrificed at 24 hour intervals to check for the disappearance of nonylphenol's estrogenic effects, i.e. the disappearance of the vitellogenin RT-PCR mRNA. Qualitative results showed a substantial decrease in the level of vitellogenin RT-PCR mRNA at 72 hours post transfer.

Rainbow trout were exposed to various levels of nonylphenol ranging from 10 to 150 ppb under flow through conditions for 72 hours. Following total RNA purification, two bands were generated by RT-PCR from nonylphenol treated rainbow trout liver. The larger band was Vitellogenin (Vg) mRNA with 827 bp (Vg59). The smaller band was the beta actin internal control mRNA, 541 bp. The vitellogenin results were verified with mRNA developed from trout ip injected with 2 μg/kg of 17 β-estradiol. No mRNA Vg band appeared in the control samples. Estrogenic effects, as evidenced by the appearance of the vitellogenin mRNA, could be seen at as low as 10 ppb of nonylphenol within 72 hours.

When rainbow trout were exposed for 72 hours to nonylphenol a maximum induction of vitellogenin was observed. After a transfer to freshwater, a substantial decrease in the level of vitellogenin was seen at 72 hours post-transfer. This rapid decrease in the level of vitellogenin could be due to either disruption of nonylphenol or the depuration of nonylphenol combined with a decrease in vitellogenin mRNA.

The work reported in this report illustrates the utility of RT-PCR in detecting low levels of vitellogenin mRNA induced by nonylphenol in rainbow trout liver. Using this method vitellogenin induction by nonylphenol would be detected after two to four days of flow-through exposure to nonylphenol.

An important finding in this study is that the concentration response curve for nonylphenol induction of vitellogenin mRNA is far to the left of the concentration lethality curve for nonylphenol. This observation indicates that the potency of nonylphenol for its estrogenic effect is much greater than its lethal effect. These *in vivo* studies indicate that nonylphenol is apt to be estrogenic in the environment at concentrations which are not lethal and tend to confirm the environmental hazard of nonylphenol.

B. Uptake, Bioaccumulation and Metabolism of Nonylphenol

1) Uptake, bioaccumulation and depuration of nonylphenol

NP isomers were radiosynthesized using n-1-nonene and ^{14}C-RUL-phenol in the presence of a catalyst. This radiosynthesis produced three major isomers, 2-(4-hydroxyphenyl)-nonane, 3-(4-hydroxyphenyl)-nonane, and 4-(4-hydroxyphenyl)-nonane. Rainbow trout were exposed to this nonylphenol mixture at 18 and 36 ppb in water to study its distribution, persistence and bioaccumulation. Nonylphenol or its metabolites were distributed throughout the body of rainbow trout including liver, muscle and fat. The apparent bioaccumulation factor in viscera and carcass ranged from 40 in carcass to 100 in viscera. At the peak of uptake, rainbow trout were transferred to fresh water to investigate the half-life of ^{14}C in various tissues. The half-life in

muscle and fat was found to be approximately 19-20 hours. Biliary excretion of ^{14}C was appreciable, and most of the ^{14}C in bile were the glucuronide congugates of 3 major metabolites.

2) *In vitro* metabolism of ^{14}C-nonylphenol

Incubation of trout hepatic microsomes with NADPH in the ^{14}C-nonylphenol isomer mixture resulted in the production of three radiolabeled metabolites whose mobility of silica TLC was similar to the deglucuronidated metabolites recovered from exposed rainbow trout bile. Metabolism of the ^{14}C-nonylphenol isomers by trout hepatic microsomes was inhibited by omission of NADPH and by addition of a P-450 inhibitor, piperonyl butoxide, to the incubations. Analysis of the metabolites extracted from the microsomal incubations using gas chromatography-mass spectroscopy indicated that the parent isomers had been hydroxylated in the C_8 position on the nonane chain to give three-omega -1 hydroxylated metabolites of each of the three parent ^{14}C-nonylphenols in the original mixture.

C. Mechanistic Studies on the Estrogenic Activity of Nonylphenol

1) Post-transcriptional regulation of vitellogenin mRNA

Vitellogenin expression driven by estrogen has been becoming a model for studying estrogenic effects in aquatic species. For the first time, we showed evidence that, without stimulation, vitellogenin mRNA precursor is expressed in both male and female immature fish. After 4 h, in fish treated with estradiol, the vitellogenin mRNA was synthesized and the precursor mRNA began to disappear. The environmental chemical, nonylphenol, showed the same effect on the vitellogenin gene expression as estrogen. It is suggested that estrogen and nonylphenol may be involved in a post-transcriptional regulation process--possibly in the initiation of pre-mRNA splicing.

2) Markers of estrogenic activity in human breast cancer cells

It has been well documented that estrogen is a facilitator of human breast cancer development and environmental estrogens such as NP have recently been under intense investigation. The reason we used the expression of PS2, MUC1 and estrogen receptor (ER) genes as markers of estrogenic effects is based on 1) PS2 is an estrogen induced gene product first identified in the MCF-7 human breast cancer cell line after estrogen treatment, but not found in normal breast tissue nor in any other cultured human cell lines. 2) MUC1 is a member of a family of high molecular weight, highly glycosylated mucin glycoproteins found in the membranes of human epithelial cells. Dysfunction of mucin, such as MUC1 overexpression, is believed to play important roles in mammary gland tumorigenesis. MUC1 expression is also induced by estrogen. 3) It is widely accepted that estrogen regulates target gene expression through the specific binding of estrogen receptor. The ER/estrogen complex is able to modulate transcriptional activity by interacting with the estrogen responsive element in target promotor genes. ER gene expression is also affected by estrogen.

The time course of mRNA expressions were detected after NP (10uM) and estradiol (E2)(0.1μM) treatments using RT-PCR technique. The products were run on a 1.6% agarose gel and quantitated with the FluorImager. 1) NP induced pS2 mRNA after a two hour exposure, similar to E2 induction. At 24 hours, the total RNA was increased 2.4 and 4.2-fold, respectively, with NP and E2 treatment, indicating possible induction of MCF-7 cell growth. 2) The highest

level of MUC1 mRNA seen after NP treatment was at 2 hours (10-fold vs. control), which was reduced to only 42% of control at 48 hr. E2 treatment resulted in a gradual increase in MUC1 expression; double the control at 2 hr, 5-fold increase at 24 hr and increased by a factor of 10 over control at 48 hrs. This indicates that NP may stimulate MUC1 expression by a different mechanism than E2. 3) NP affected ER expression in the same manner as MUC1; the highest level of expression occurred at 2 hr, but expression was gradually reduced to control level by 48 hr. In contrast, E2 stimulated ER expression in a similar manner as pS2; the highest level was at 2 hr and expression remained elevated through the 48 hr point.

NP is an estrogenic compound that alters PS2, MUC1 and ER gene expression, possibly by a different mechanism than E2. According to recent literature, however, the mechanism by which E2 and NP regulate gene expression is still unclear, as is the role of ER in the process. Since induction begins after a 2 hour exposure, it has been suggested that a post-transcriptional regulation process may be invovled. The link between aberrant MUC1, PS2 and ER expression and the development of breast cancer also needs to be elucidated through further investigation.